

**EXPRESSION AND PURIFICATION OF INTERNALIN A LIGAND  
FOR INTERNALIZATION STUDIES ON NON-PROFESSIONAL  
PHAGOCYtic EPITHELIAL CELLS**

A Thesis  
Presented to  
The Academic Faculty

by

Natalie Haddad

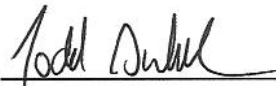
In Partial Fulfillment  
of the Requirements for the Bachelors of Science Degree  
Biology in the  
College of Sciences

Georgia Institute of Technology  
December 2011

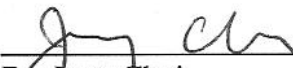
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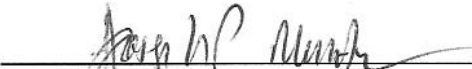
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## **ACKNOWLEDGMENTS**

The project discussed in this thesis was carried out in Dr. Todd Sulchek's laboratory in the Parker H. Petit Institute of Bioengineering and Biosciences on the Georgia Institute of Technology campus. Project funding was provided by the 2011 Petit Undergraduate Research Scholarship Program. I would like to thank my principal investigator, Dr. Sulchek, and my graduate student mentor, Patricia Pacheco for their visions of the project and continued support throughout the project. I would also like to thank my Honors Research Thesis professor Dr. Jung Choi of the Biology Department at Georgia Tech for serving as my Biology faculty co-advisor on the project. Lastly, I would like to thank Dr. Youn-Hi Woo, one of the researchers of an important preceding study, for providing the pGEX-6p-1-InlA plasmid and assistance for some of the methods involved in this project.

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## LIST OF SYMBOLS AND ABBREVIATIONS

$A_{260}$	Absorbance reading from spectrophotometer at 260 nanometer wavelength
$A_{600}$	Absorbance reading from spectrophotometer at 600 nanometer wavelength
AmpR	Ampicillin antibiotic resistance
BL21	One Shot BL21(DE3)pLysS expression competent <i>E. coli</i> cells
CamR	Chloramphenicol antibiotic resistance
DH5 $\alpha$	MAX Efficiency DH5 $\alpha$ F' IQ competent <i>E. coli</i> cells
E-cadherin	Epithelial cadherin surface receptor
<i>E. coli</i>	<i>Escherichia coli</i> bacteria
GST	Glutathione S-transferase
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i> bacteria
InlA	Internalin A protein
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside

## ABSTRACT

Infection by intracellular pathogens is a growing concern as these microorganisms can cross their host's cellular barrier and use the host's own cellular proteins for replication, making treatment very difficult (Kenneth et al., 2008). A further restriction for treatment is that a proposed drug must be able to cross the same barriers in order to reach these pathogens. Our study investigates the same protein used by a particular intracellular pathogen, *Listeria monocytogenes*, as a possible internalization method for intracellular delivery of materials or drugs. A pGEX-6p plasmid vector containing a gene for the bacterial surface protein Internalin A (InlA) and a glutathione *S*-transferase (GST) tag, was restored and transformed into *Escherichia coli* MAX Efficiency DH5 $\alpha$ F' IQ competent cells in order to increase insert stability and DNA quality. The plasmid was then purified and subsequently transformed into One Shot BL21(DE3)pLysS expression competent *E. coli* cells. Thereafter, the InlA-GST fusion protein was expressed in the BL21 cells using Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). It has been shown, through selective plating and gel electrophoresis, that the plasmid was successfully transformed and purified. Our next steps will be to purify the InlA-GST fusion protein using glutathione affinity based chromatography, cleave and remove the GST tag from the InlA protein of interest, fluorescently label InlA using Alexa Fluor, and bind InlA to microbeads of various sizes in a range of densities. We will then carry out internalization assays of the InlA-coated beads in different epithelial cell lines to demonstrate the versatility of this method as a possible material or drug delivery option.



# **CHAPTER 1**

## **INTRODUCTION**

Virtually all cells, including unicellular organisms, live in the vicinity of other cells. Communication with their neighbors is a fundamental property that influences their every function. As a consequence, all cells have signaling mechanisms involved in metabolic processes, growth and differentiation, synthesis and secretion of proteins, and responding to environmental conditions (Lodish et al., 2008). Signaling is also extremely important in the immune response to harmful pathogenic microorganisms and viruses. Immune-system cells sense changes in the extracellular environment and are activated through their cell-surface receptors. This can take the form of specific antigen recognition as in T- and B-lymphocyte activation or general pathogen pattern recognition found in innate immune cells such as macrophages, which internalize and destroy pathogens through a process known as phagocytosis (Kenneth et al., 2008). The phagocytosis process is well studied in “professional” phagocytic cells such as macrophages; however, pathogens are often internalized by “non-professional” phagocytes as well, including epithelial cells. The mechanisms underlying the process in the later are not as well understood (Blanchette et al., 2009; Lecuit, 2005).

It is known that pathogens that are internalized by non-professional phagocytes can take advantage of the host’s cell-surface receptors and signaling pathways which result in internalization of the pathogen. Once inside, the pathogen can take advantage of more of the host’s cellular proteins and cytoskeleton, aiding its movement and replication, (Pizarro-Cerda et al., 2004) and sometimes evade normal immune responses

(Kenneth et al., 2008). The costs that these pathogens impose on their hosts are evident in harmful diseases that are caused by intracellular pathogens (Barbuddhe & Chakraborty, 2009). Because of the nature of infection inside of the cell, treatment of these diseases can be difficult, as proposed drugs would also need to cross through the cellular membrane to combat the pathogen. Further investigation of the mechanisms and proteins employed by the very pathogens that are internalized may prove valuable in developing novel drug therapies. A model intracellular pathogen is *Listeria monocytogenes*, a bacterium that induces its internalization by binding one of its surface proteins, Internalin A (InlA), to an epithelial host cell's E-cadherin receptor (Bonazzi et al., 2009).

Under the guidance of Dr. Todd Sulchek and graduate student Patricia Pacheco, I have generated the InlA protein for phagocytosis studies on non-professional phagocytic epithelial cells. I achieved this by first transforming *Escherichia coli* MAX Efficiency DH5 $\alpha$ F'IQ competent cells (Invitrogen, Carlsbad, CA), with a pGEX-6p-1-InlA ampicillin resistant (AmpR) plasmid, an extra-chromosomal circular piece of DNA containing a gene for InlA and an attached (glutathione *S*-transferase) GST tag (Blanchette et al., 2009). I then purified the plasmid from the DH5 $\alpha$  cells using a QIAGEN plasmid purification midi kit (QIAEGN, Valencia, CA), and subsequently transformed the plasmid once again into One Shot BL21(DE3)pLysS expression competent *E. coli* cells (Invitrogen, Carlsbad, CA). The production of the InlA-GST fusion protein was then expressed using Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG).

MAX Efficiency DH5 $\alpha$ F'IQ competent *E. coli* cells were used because they contain recA1 and endA1 mutations which increase insert stability and improve the quality of plasmid DNA (Invitrogen, 2006). One Shot BL21(DE3)pLysS expression

competent *E. coli* cells were used to achieve high-efficiency expression of the InlA-GST fusion protein gene, which is under the control of a T7 promoter. The BL21 strain contains a DE3 lysogen carrying a gene for T7 RNA polymerase under the control of the lacUV5 promoter, which ultimately means that expression of T7 RNA polymerase and subsequent InlA-GST expression requires Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). In this way, I was able to control when InlA expression was to be carried out during the experiment by addition of IPTG to liquid culture of BL21(DE3)pLysS-pGEX-6P-1-InlA-1 *E. coli* cells. The BL21 strain used in this study also contains a pLysS plasmid, which contains a T7 lysozyme gene responsible for lowering background expression level of InlA under control of the T7 promoter. After IPTG is added, however, the T7 lysozyme does not affect the expression of InlA-GST (Invitrogen, 2010). The pLysS plasmid also contains a chloramphenicol resistance (CamR) gene and a p15A origin, allowing it to be compatible with plasmids containing pMB1 origin (Invitrogen, 2010), including the pBR322- derived pGEX-6P plasmid used in this study. The pGEX-6P plasmid used carries an ampicillin resistance (AmpR) gene, which allows bacteria harboring the pGEX-6P plasmid to grow in the presence of ampicillin antibiotic (Blanchette et al., 2009). The pGEX-6P plasmid is a glutathione *S*-transferase (GST) expressing vector with a GST sequence adjacent to the InlA gene so that an InlA-GST fusion protein was expressed when IPTG was added. This GST tag allows the fusion protein containing the protein of interest, InlA, to be purified from solution by glutathione affinity based chromatography, due to the affinity of GST for glutathione substrate (Blanchette et al., 2009).

The future steps in this project will be to purify the InlA-GST fusion protein from the BL21 cells using glutathione affinity based chromatography, cleave and remove the GST tag from the InlA protein, fluorescently label InlA with Alexa Fluor dye, then bind InlA to different sizes of microbeads in varying densities by passive adsorption. Finally, the InlA functionalized beads will be administered to several epithelial cell line cultures in vitro to induce phagocytosis of the beads. Cell internalization assays will be conducted in order to compare the effects of ligand density, bead size, and eukaryotic target cell versatility and analyzed quantitatively using flow cytometry, and qualitatively using fluorescent microscopy. Previous knowledge and studies, discussed in the literature review, provide insight into the importance of our research.

## CHAPTER 2

### BACKGROUND INFORMATION AND LITERATURE REVIEW

Cells respond to their extracellular environment when molecules bind to their surface receptor proteins, which activate signal transduction pathways inside the cells (Kenneth et al., 2008). A superfamily of cell surface transmembrane glycoproteins which participate in cellular adhesion, recognition, and signaling includes the cadherin receptors. Classical cadherins, known as Type 1 cadherins, are known to associate with the actin cytoskeleton and are found in most solid tissues of the body including the epithelial, neural, and vascular endothelial cells (Yap & Goodwin, 2004). Interactions between cells and the extracellular matrix are important in allowing cells to aggregate into tissues and also for bidirectional communication between the exterior and the interior of cells. Many biological processes such as cell survival, proliferation, differentiation, and migration depend on this type of cellular communication (Lodish et al., 2008); however, this system can also be exploited by harmful pathogens. One of these prominent intracellular pathogens is *Listeria monocytogenes*, the bacterium responsible for a highly fatal foodborne disease listeriosis (Barbuddhe & Chakraborty, 2009). *L. monocytogenes* exploits the host cell's epithelial cadherin receptor, E-cadherin, as well as its actin cytoskeleton in the cytosol, allowing the bacteria to move around freely within the host cell (Dramsi et al., 1998). This bacterium uses a surface protein, Internalin A (InlA), to interact with the host cell's E-cadherin receptor. This activates a cellular signaling pathway that results in internalization of the bacterium into endothelial eukaryotic cells (Bonazzi et al., 2009). A previous study ascertained the crystalline structures of the InlA

functional domain both alone and in conjunction with the extracellular, N-terminal domain of E-cadherin. E-cadherin is surrounded and recognized by the leucine-rich repeat domain of InlA. Researchers in this study were able to probe individual interactions by mutagenesis and analytical ultracentrifugation, including a major determinant for human susceptibility to *L. monocytogenes* infection, Pro16 of human E-cadherin, which is important for intermolecular recognition (Schubert et al., 2002).

The dangers of *L. monocytogenes* are highlighted by the fact that it is found in a variety of environmental sources, including soil, water, sewage waste, food, and human and animal feces (Barbuddhe & Chakraborty, 2009). In fact, a single *Listeria* outbreak was responsible for at least 29 deaths in the United States in the year 2011, due to consumption of contaminated Rocky Ford-brand cantaloupes grown and distributed by Jensen Farms in Colorado. According to the Centers for Disease Control and Prevention, this incident is the deadliest food-borne illness outbreak in the United States since 1924. With 139 reported illnesses so far, the death toll is even still rising weeks after the cantaloupe recall due to the long incubation period of *Listeria*, up to two months before causing any sign of illness. This is because *Listeria* is able to thrive and proliferate successfully in epithelial cells over a long time and evade the immune system response (Weise, 2011). One of the ways in which *Listeria* can evade the immune system response is by preventing the phagosome containing *Listeria* from fusing with a lysosome, which is a crucial step in the phagocytosis process. This step most likely enhances usual host defense because the phagolysosome created is very acidic and contains hydrolytic enzymes, both properties of which inhibit microbial growth (Blanchette et al., 2009).

Infection by intracellular pathogens is a vital concern as these microorganisms can use their host's own cellular proteins for replicating and spreading, making treatment very difficult (Kenneth et al., 2008). A further restriction for treatment is that a proposed drug must be able to cross the host's cellular barriers in order to reach these pathogens. The key to an efficient internalization option for drug delivery may lie in the machinery used by the very pathogens that the drug seeks to combat. Another previous study, in which the principle investigator of this current project, Dr. Sulchek, was involved in, has shown that microparticles functionalized with InlA can be internalized by non-phagocytotic epithelial cells. This study developed a method with which to measure and decouple particle internalization, phagosomal acidification, and phagosomal/endosomal/lysosomal fusion in Madin-Darby Canine Kidney (MDCK) and Caco-2 epithelial cells. They independently measured the rates of each step by combining the InlA-coated beads with antibody quenching dye, a pH sensitive dye, and an endosomal/lysosomal dye. They found the time scales for each step to be 23-32 minutes, 3-4 minutes and 74-120 minutes, respectively, for MDCK and Caco-2 epithelial cells (Blanchette et al., 2009). This study is important because while phagocytosis has been assessed in "professional" phagocytic cells of the immune system such as polymorphonuclear leukocytes, (PMNs), monocytes, neutrophils and macrophages, little has been done in the past to examine the process in non-professional phagocytic epithelial cells (Blanchette et al., 2009), which are often the targets of intracellular pathogens.

The study done by Blanchette et al, especially, was used as a reference for most of the methods in our project. In fact, the pGEX-6p-1-InlA plasmid used in this current project was recovered from stored samples remaining from the previous project. The

previous researchers amplified the extracellular domain of InlA (the N8 terminal 500 residues) from genomic DNA of *L. monocytogenes*, ligated it into a TOP-2.1 vector (Invitrogen, Carlsbad, CA), and transformed it into *Escherichia coli* MAX Efficiency DH5 $\alpha$  F' IQ competent cells. The researchers selected the amplified product for the insert of appropriate direction and digested it with EcoRI and BamHI. Finally, the insert was ligated into a GST expression vector, pGEX-6p-1 (Stratagene, La Jolla, CA). They then transformed the final plasmid construct, pGEX-6p-1-InlA once again into DH5 $\alpha$  *E. coli* and confirmed the final sequence (Blanchette et al., 2009).

As well as being able to enter, survive, and multiply in both phagocytic and non-phagocytic cells, *L. monocytogenes* is also versatile in that it can cross the intestinal barrier, blood-brain barrier, and fetoplacental barrier in humans (Lecuit, 2005). Such an organism with a versatile, entry-inducing protein can surely provide great insight into versatile drugs which could enter a vast range of infected cells. Applying this pathogenic mimicry system to developing drugs is an important area of research that warrants further consideration because epithelial cells are often targets of intracellular pathogens such as *L. monocytogenes* (Schubert et al., 2002). Although it is known that InlA functionalized microparticles are digested by some non-phagocytotic epithelial cells, it would be useful to direct further research in comparing internalization rates of a large variety of non-phagocytotic cells in order to demonstrate the range of target cell versatility of this method as a possible drug delivery option. With proper knowledge of mechanisms used by intracellular pathogens, drugs can be developed which can be easily internalized by various types of infected cells, leading to better universal treatment of intracellular diseases.



## CHAPTER 3

### MATERIALS AND METHODS

#### Plasmid Preparation

The pGEX-6p-1-InlA plasmid was created according to the protocol in Blanchette et al., 2009, then freeze dried and stored at -20°C on filter paper.

#### Plasmid Recovery and Initial DH5 $\alpha$ Transformation

The plasmid, which also contains a gene for ampicillin resistance (AmpR), was recovered by incubating the filter paper containing the plasmid in TE buffer (10 mM Tris-HCl, 1 mM EDTA) obtained from Invitrogen (Carlsbad, CA). *Escherichia coli* MAX Efficiency DH5 $\alpha$ F' IQ competent cells were purchased from Invitrogen (Carlsbad, CA), and transformed with the pGEX-6p-1-InlA plasmid according to the manufacturer's protocol: The MAX Efficiency DH5 $\alpha$ F' IQ competent cells were thawed on wet ice then mixed with the pGEX-6p-1-InlA plasmid TE buffer solution. As a negative control, another sample of DH5 $\alpha$  competent cells was mixed with control M13mp19RF DNA solution, also from Invitrogen (Carlsbad, CA). The cells were incubated on ice for 30 minutes, heat-shocked for 45 seconds in a 42°C water bath, and then incubated on ice for an additional two minutes. S.O.C. medium and DH5 $\alpha$ F' IQ lawn cells (Invitrogen, Carlsbad, CA), were added to the competent cells before plating on LB agar (10 g SELECT Peptone 140, 5 g SELECT Yeast Extract, 5 g sodium chloride per liter media; Invitrogen, Carlsbad, CA), plates containing ampicillin (50 $\mu$ g/ml), inverting, and incubating at 37°C overnight. The next day the plates were moved to the 4°C refrigerator in order to slow bacterial growth. Later, a single colony was picked and re-streaked in a four-way streak

manner onto a fresh LB plus ampicillin (50µg/ml) plate, incubated at 37°C overnight and transferred to the 4°C refrigerator once again.

### **Plasmid Purification**

A Plasmid Purification Midi Kit was purchased from QIAGEN (Valencia, CA) and the accompanying manufacturer's protocol was carried out to purify the InlA plasmid from the transformed DH5α *E. coli* cells:

A single colony was picked from the selectively streaked DH5α plate and a starter culture was inoculated in 5 ml LB medium containing ampicillin (50µg/ml). The culture was incubated for approximately 8 hours at 37°C with vigorous shaking of approximately 300 rpm. The starter culture was diluted 1/1000 into ampicillin (50µg/ml) selective LB medium (100 ml medium, 100 µl starter culture) and grown at 37°C overnight with vigorous shaking of approximately 300 rpm. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The bacterial pellet was re-suspended in 4ml of kit-provided Buffer P1. A volume of 4ml Buffer P2 was added, mixed thoroughly by vigorously inverting the sealed tube 6 times, and incubated at room temperature for 5 minutes before 4ml of chilled Buffer P3 was added. Immediately upon addition of Buffer P3, the solution was mixed thoroughly by vigorously inverting 6 times then allowed to incubate on ice for 15 minutes. The solution was centrifuged at 20,000 x g for 30 minutes at 4°C, and the supernatant containing the plasmid was removed promptly. A 240 µl sample of the cleared lysate was saved for analytical gel sample in order to determine whether growth and lysis conditions were optimal. A QIAGEN-tip 100 was equilibrated by applying 4 ml of Buffer QBT and the column was allowed to empty by gravity flow. The previously isolated supernatant was applied to the QIAGEN-tip and allowed to enter

the resin by gravity flow. A 240 µl sample from the flow-through was saved for an analytical gel in order to determine the efficiency of DNA binding to the QIAGEN Resin. The QIAGEN-tip was then washed with 2 x 10 ml Buffer QC. A sample of 400 µl was taken from the combined wash fractions and saved for analytical gel. The DNA was eluted with 5 ml Buffer QF and a sample of 100 µl was saved for an analytical gel. The DNA was precipitated by adding 3.5 ml (0.7 volume) room-temperature isopropanol to the eluted DNA. It was then mixed and centrifuged immediately at 15,000 x g for 30 minutes at 4°C and the supernatant was carefully decanted. The DNA pellet was washed with 2ml of room-temperature 70% ethanol and centrifuged at 15,000 g for 10 minutes. The supernatant was carefully decanted without disturbing the pellet. The pellets were then air dried for 10 minutes and the DNA was re-dissolved in 2 ml TE buffer. Absorbance readings were taken at 260 nm for determination of plasmid yield. Finally, the plasmid-TE buffer solution was split into four working aliquots of 0.5ml and stored in a -20°C freezer until it could be retransformed into One Shot BL21(DE3)pLysS expression competent *E. coli* cells according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

### **Agarose Gel Electrophoresis**

Samples were taken throughout the midikit purification procedure and analyzed using gel electrophoresis. A 1% agarose gel was prepared and placed in the gel electrophoresis tank with 1X TBE buffer. Lane 1 was loaded with 1µl 1Kb Plus DNA ladder (Invitrogen, Carlsbad, CA), 3 µl TE buffer, and 1 µl BlueJuice Gel Loading Buffer (10X) (Invitrogen, Carlsbad, CA). The next 5 lanes were loaded with 4µl samples from the cleared lysate, flowthrough, wash fractions, elute, and purified plasmid from QIAGEN midi kit protocol,

each mixed with 1µl BlueJuice Gel Loading Buffer. The gel was run at 100 volts for approximately 45 minutes until the blue dye reached nearly the end of the gel. The gel was stained with 0.5 µl/ml Ethidium bromide water solution in the dark with gentle agitation for approximately 30 minutes. The gel was then de-stained with De-ionized water in the dark with gentle agitation for approximately 10 minutes before being transferred to a UV transilluminator for imaging.

### **One Shot BL21(DE3)pLysS Transformation**

One Shot BL21(DE3)pLysS competent *E. coli* cells were purchased from Invitrogen (Carlsbad, CA), and transformed with the purified pGEX-6p-1-InlA plasmid according to the manufacturer's protocol:

Two vials (50µl each) of BL21 cells were thawed on wet ice. One of the working aliquots of purified plasmid solution was thawed and the absorbance was taken again for accuracy of transformation. Approximately 2.0µl of the TE buffer plasmid solution, containing approximately 8.0 ng of pGEX-6p-1-InlA plasmid DNA was added to one of the vials of BL21 cells. Approximately 1µl of pUC19 control plasmid solution, containing approximately 10pg of plasmid was added to the other BL21 vial. Each vial was mixed separately by gentle tapping. The vials were incubated on ice for 30 minutes, heat shocked for 30 seconds in a 42°C water bath, then immediately placed on ice again. Each vial had added to it 250µl of room temperature S.O.C medium then placed in a shaking incubator at 37°C and 225 rpm for 1 hour. Samples of 50µl and 150µl of BL21(DE3)pLysS-pGEX-6P-1-InlA-1 *E. coli* cells transformed with the InlA plasmid were plated on two separate LB agar plates each containing ampicillin (50µg/ml) and chloramphenicol (34µg/ml). Two 50µl samples of BL21(DE3)pLysS cells transformed

with the pUC19 control plasmid were plated on two separate LB agar plates both containing ampicillin (50µg/ml) and chloramphenicol (34µg/ml). In order to check for phage contamination, 50µl of un-transformed BL21(DE3)pLysS *E. coli* cells were plated on an LB agar plate containing no antibiotics. In order to verify for the absence of ampicillin resistance contamination, 50µl of un-transformed BL21(DE3)pLysS *E. coli* cells were plated on an LB agar plate containing ampicillin (50µg/ml). Finally, in order to verify the presence of the pLysS plasmid in the BL21(DE3)pLysS *E. coli* cells which confers chloramphenicol resistance and expresses the T7 lysozyme, 50 µl of un-transformed BL21(DE3)pLysS *E. coli* cells were transformed on an LB agar plate containing chloramphenicol (34µg/ml). Each plate was inverted and incubated at 37°C overnight.

### **InlA-GST Expression**

Colonies were selected from the 50µl spread plate of BL21(DE3)pLysS-pGEX-6P-1-InlA-1 *E. coli* cells and diluted into liquid LB media containing 50µg/ml ampicillin and 34µg/ml chloramphenicol. Four 5ml cultures were made containing 4 colonies each. The cultures were incubated at 37°C and 225 rpm overnight in 50ml centrifuge tubes. The following day, the overnight cultures were diluted. Two of the tubes were diluted into 250 ml of LB media each in separate 1 liter flasks containing ampicillin (50µg/ml) and chloramphenicol (34µg/ml), and the other two tubes were diluted together in 500 ml of LB media with ampicillin (50µg/ml) and chloramphenicol (34µg/ml) in a 2 liter flask. The cultures were incubated at 37°C and 225 rpm, and after 2 hours the absorbance at 600 nm ( $A_{600}$ ) was checked from each flask. When  $A_{600}$  was in the 0.04-0.06 range for each flask, InlA-GST expression was induced by adding 2mM IPTG (0.5ml of 1M IPTG stock

solution per 250ml of culture). The cells were allowed to grow at 37°C and 225 rpm for an additional 4 hours until growth was stopped by placing on ice. The cultures were then transferred to six 50 ml centrifuge tubes and centrifuged at 4,500 rpm, 4 °C for 30 minutes in 4 rounds. The supernatant was discarded after each round and the final cell pellets were frozen at -20° C, until lysis and purification of InlA-GST could be carried out.

## CHAPTER 4

### RESULTS

#### Plasmid Recovery and Initial DH5 $\alpha$ Transformation

Several white colonies were observed in the LB ampicillin (50 $\mu$ g/ml) agar plate spread with MAX Efficiency DH5 $\alpha$ F'IQ competent *E. coli* cells transformed with the pGEX-6p-1-InlA ampicillin resistant plasmid (figure 1.0 A), while the DH5 $\alpha$  *E. coli* cells transformed with the control M13mp19RF plasmid DNA did not form colonies in the presence of ampicillin (50 $\mu$ g/ml) LB agar (figure 1.0 B).

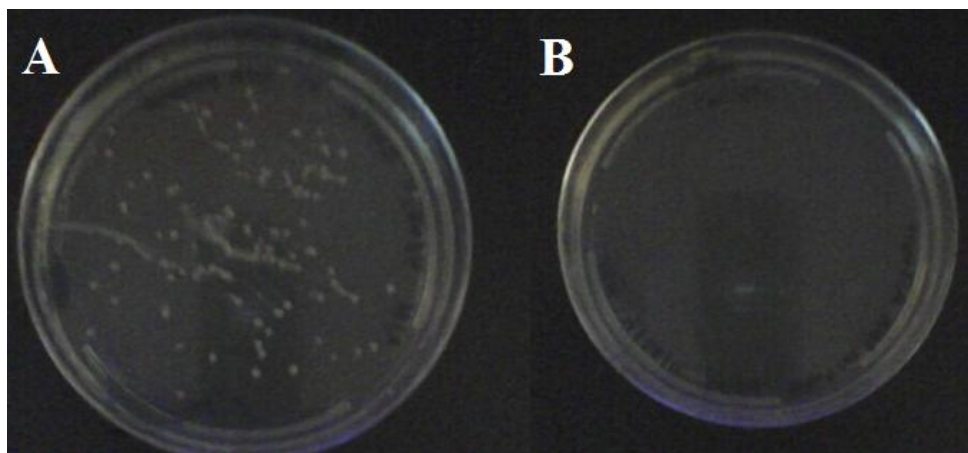


Figure 1.0. *Escherichia coli* MAX Efficiency DH5 $\alpha$ F'IQ competent cells transformed with pGEX-6p-1-InlA plasmid (A) and with control M13mp19RF plasmid (B). MAX Efficiency DH5 $\alpha$ F'IQ competent cells were thawed on wet ice, mixed with pGEX-6p-1-InlA ampicillin resistant plasmid TE buffer solution (A) or with control M13mp19RF DNA solution (B), incubated on ice for 30 minutes, heat-shocked for 45 seconds in a 42°C water bath, and incubated on ice for two minutes. Cells were added to S.O.C. medium and DH5 $\alpha$ F'IQ lawn cells, plated on LB agar plates containing ampicillin (50 $\mu$ g/ml), inverted, and incubated at 37°C overnight.

## Plasmid Purification

DH5α *E. coli* cells transformed with pGEX-6p-1-InlA plasmid were cultured and pelleted using centrifugation. Alkaline lysis was used to break open the bacterial cells and expose the DNA. The plasmid DNA was bound to QIAGEN Anion-Exchange Resin under low-salt and pH conditions. The RNA, proteins, dyes, and low molecular weight impurities were removed by medium salt wash. The plasmid DNA was eluted in a high-salt buffer and then concentrated and de-salted by isopropanol precipitation. The purified plasmid was then re-dissolved in 2 ml TE buffer and the absorbance of the sample was taken using spectrophotometer at 260 nm ( $A_{260}$ ) in order to determine the yield of purified plasmid. The average absorbance reading for the plasmid solution was  $A_{260}=0.05$ . The concentration of purified plasmid in the TE buffer was found to be approximately 2.5µg/ml (Equation 1.0), and therefore the total yield of plasmid purified from the procedure was approximately 5µg (Equation 2.0).

$$A_{260} = 1 \Rightarrow \frac{50 \mu g}{ml} \quad A_{260} \approx 0.05 \Rightarrow 0.05 \times \frac{50 \mu g}{ml} \approx 2.5 \mu g \text{ purified plasmid per ml TE buffer}$$

Equation 1.0. Calculation of purified plasmid concentration per ml TE buffer after QIAGEN midi kit purification of pGEX-6P-1-InlA-1 plasmid from DH5α *E. coli* cells.

$$\frac{2ml}{ml} \times \frac{2.5 \mu g}{ml} \approx 5 \mu g \text{ purified pGEX-6P-1-InlA-1 plasmid}$$

Equation 2.0. Calculation of total yield of pGEX-6P-1-InlA-1 plasmid purified using QIAGEN midi kit.



## Agarose Gel Electrophoresis

The agarose gel electrophoresis image shows clear bands representing the 1Kb Plus DNA ladder (Figure 2.0 A) and samples 1-4 taken from the QIAGEN plasmid purification midi kit procedure including the cleared lysate containing supercoiled and open circular plasmid DNA and degraded RNA (Figure 2.0 B); flow-through fraction showing supercoiled (lower band) and open circular form (upper band) of the plasmid with denatured supercoiled DNA migrating just below the supercoiled form (Figure 2.0 C); Wash fractions (Figure 2.0 D); and the elute containing pure plasmid DNA with no other contaminating nucleic acids (Figure 2.0 E). The final purified plasmid stored in TE buffer also showed a clear band around the same distance as the elute as well as a faint upper band representing open circular DNA between 2.0kb and 5.0kb (Figure 2.0 F).

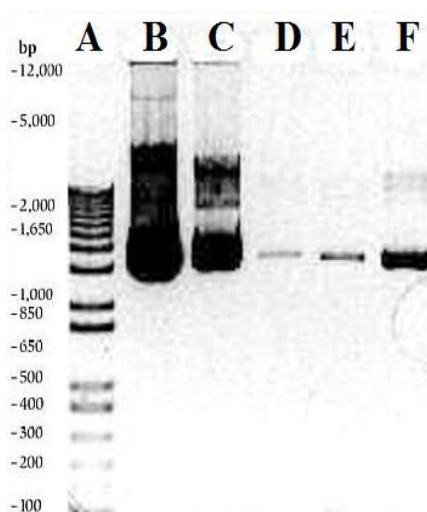


Figure 2.0. Agarose gel electrophoresis analysis from plasmid purification. DH5 $\alpha$  *E. coli* cells with InlA plasmid were cultured and pelleted using centrifugation. Alkaline lysis was used to break open the bacterial cells and expose the DNA. The plasmid DNA was bound to QIAGEN Anion-Exchange Resin under low-salt and pH conditions. The RNA, proteins, dyes, and low molecular weight impurities were removed by medium salt wash. The plasmid DNA was eluted in a high-salt buffer and then concentrated and de-salted by isopropanol precipitation. (A) 1Kb Plus DNA ladder (Invitrogen). (B) Sample 1: Cleared lysate containing supercoiled and open circular plasmid DNA and degraded RNA. (C) Sample 2: Flow-through fraction. Supercoiled (lower band) and open circular form (Upper band) of the plasmid with denatured supercoiled DNA migrating just below the supercoiled form. (D) Sample 3: Wash fractions. (E) Sample 4: Elute containing pure plasmid DNA with no other contaminating nucleic acids. (F) Final purified plasmid supercoiled form (lower dark band). Faint upper band shows open circular plasmid between 2kb and 5kb. The pGEX plasmid is around 4.9Kb.

### **One Shot BL21(DE3)pLysS Transformation**

Approximately 8.0 ng of the purified ampicillin resistant pGEX-6P-1-InlA-1 plasmid was transformed into One Shot BL21(DE3)pLysS expression competent *E. coli* cells.

Volumes of 50µl and 150µl of culture were plated onto LB agar plates containing ampicillin (50µg/ml) and chloramphenicol (34µg/ml). Both plates yielded abundant white colonies with the 150µl culture displaying a much denser area of growth (Figure 3.0 B) than the 50µl culture (Figure 3.0 A). Approximately 10 pg of control ampicillin resistant pUC19 plasmid was transformed into One Shot BL21(DE3)pLysS expression competent *E. coli* cells and a volume of 50µl culture was plated onto two LB agar plates containing ampicillin (50µg/ml) and chloramphenicol (50µg/ml). The first plate had 6 white colonies (Figure 3.0 C), and the second plate had 4 white colonies, yielding an average of 5 pUC19 control colonies for the transformation efficiency. 50 µl of un-transformed BL21(DE3)pLysS *E. coli* cells was plated onto LB agar containing no antibiotics in order to check for phage contamination. The plate yielded a lawn of BL21 cells (Figure 3.0 E). 50 µl of un-transformed BL21(DE3)pLysS *E. coli* cells was plated onto LB agar plates containing ampicillin (50µg/ml) in order to verify for the absence of ampicillin resistance contamination. No colonies formed on the plate (Figure 3.0 F). 50 µl of un-transformed BL21(DE3)pLysS *E. coli* cells was plated onto LB agar containing chloramphenicol (34µg/ml) in order to verify the presence of the pLysS plasmid in the BL21(DE3)pLysS cells which expresses the T7 lysozyme. Abundant colonies formed on the plate (Figure 3.0 G).

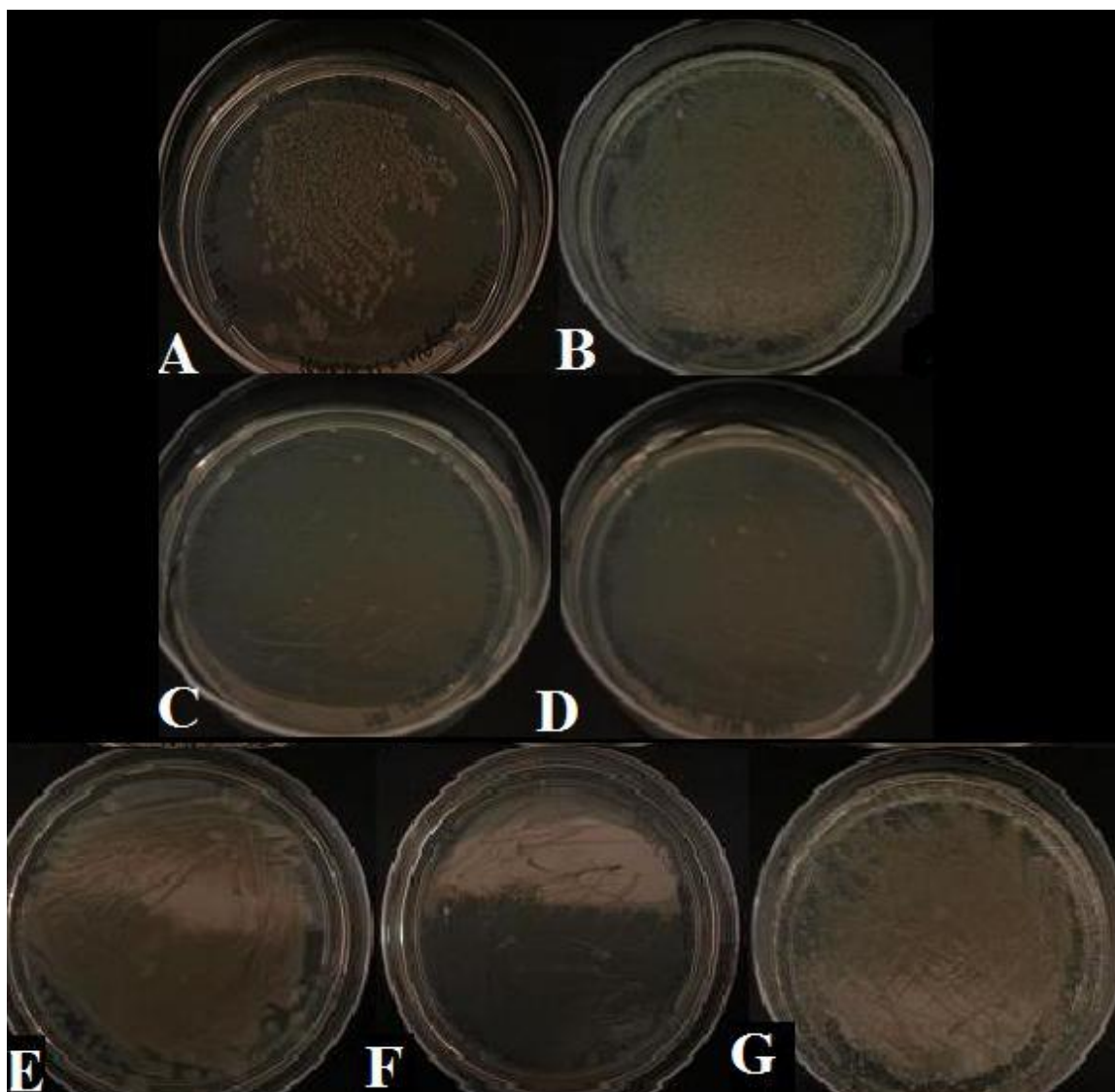


Figure 3.0. Transformation of One Shot BL21(DE3)pLysS competent *E. coli* cells (A) 50µl BL21(DE3)pLysS *E. coli* cells transformed with pGEX-6P-1-InlA-1 plasmid plated on LB agar plate containing ampicillin (50µg/ml) and chloramphenicol (50µg/ml). pLysS plasmid has chloramphenicol resistance and the InlA plasmid has ampicillin resistance. (B) 150µl BL21(DE3)pLysS *E. coli* cells transformed with pGEX-6P-1-InlA-1 plasmid plated on LB agar plate containing ampicillin (50µg/ml) and chloramphenicol (34µg/ml). (C, D) Replicates of 50µl BL21(DE3)pLysS *E. coli* cells transformed with pUC19 control ampicillin resistant plasmid plated on LB agar plates containing ampicillin (50µg/ml) and chloramphenicol (34µg/ml). (E) 50 µl un-transformed BL21(DE3)pLysS *E. coli* cells plated on LB agar containing no antibiotics. A lawn is apparent which confirms that there is no phage contamination. (F) 50 µl un-transformed BL21(DE3)pLysS *E. coli* cells plated on LB agar containing ampicillin (50µg/ml). Lack of growth verifies absence of ampicillin resistance contamination. (G) 50 µl un-transformed BL21(DE3)pLysS *E. coli* cells plated on LB agar containing chloramphenicol (34µg/ml). Growth verifies the presence of the pLysS plasmid in the BL21(DE3)pLysS cells which expresses the T7 lysozyme.

The average number of colonies of BL21 transformed with the control ampicillin resistant pUC19 plasmid was used to calculate the transformation efficiency using the calculation provided in the One Shot BL21(DE3)pLysS user manual by Invitrogen (Carlsbad, CA). The transformation efficiency proved to be  $3 \times 10^6$  transformants per  $\mu\text{g}$  of plasmid DNA (Equation 3.0).

$$\frac{5 \text{ colonies}}{10 \text{ pg transformed DNA}} \times \frac{10^6 \text{ pg}}{\mu\text{g}} \times \frac{300 \mu\text{l transformed cells}}{50 \mu\text{l plated}} = \frac{3 \times 10^6 \text{ transformants}}{\mu\text{g plasmid DNA}}$$

Equation 3.0. Calculation of transformation efficiency of One Shot BL21(DE3)pLysS expression competent *E. coli* cells using the average number of colonies of BL21 cells transformed with the control ampicillin resistant pUC19 plasmid grown on LB agar plates containing ampicillin (50 $\mu\text{g}/\text{ml}$ ) and chloramphenicol (34 $\mu\text{g}/\text{ml}$ ).

## CHAPTER 5

### DISCUSSION

It has been shown that the pGEX-6p-1-InlA plasmid was successfully restored from its freeze-dried, filter paper storage condition and transformed into the *Escherichia coli* MAX Efficiency DH5 $\alpha$  IQ competent cells (Figure 1.0). DH5 $\alpha$  cells were transformed with either the pGEX-6p-1-InlA plasmid containing a gene for ampicillin resistance, or with the control M13mp19RF plasmid which did not confer ampicillin resistance. Both types of cells were plated onto LB agar plates containing ampicillin antibiotic (50 $\mu$ g/ml). The plates were inverted, and incubated overnight at 37°C. Only the plate containing the DH5 $\alpha$  cells transformed with the pGEX-6p-1-InlA plasmid formed bacterial colonies, confirming that the ampicillin resistant plasmid was in fact transformed in the DH5 $\alpha$  cells, which would otherwise not have grown in the presence of ampicillin. This initial transformation into DH5 $\alpha$  was important to increase the stability of the InlA inserted gene and plasmid, and also to improve the quality of the DNA for following InlA-GST expression (Invitrogen, 2006). The plate containing DH5 $\alpha$  cells transformed with the control M13mp19RF plasmid did not form colonies, because the cells could not grow in the presence of ampicillin, as the control plasmid did not confer ampicillin resistance. This also demonstrated that the ampicillin antibiotic was functional and had not degraded throughout the process, and was therefore a reliable means to selectively grow cells containing the plasmid of interest.

The results of the agarose gel electrophoresis show that the pGEX-6p-1-InlA was successfully purified from the DH5 $\alpha$  cells as the same clearly defined band located

between 1.0kb and 1.65kb became more pronounced from the elution sample (Figure 2.0. E), to the concentrated final purified product in TE buffer (Figure 2.0. F). The distinct upper band found in both Figure 2.0 E and F between 2.0kb and 5kb represents the open circular form of the plasmid. The pGEX plasmid is approximately 4.9 kb, but since the DNA ladder used was linear DNA, the base pair measurement of the open circular plasmid DNA is closer to the actual size of the plasmid than the base pair measurement of the closed circular plasmid DNA. When the final purified plasmid was re-dissolved in 2ml TE buffer, absorbance readings were taken to ascertain the total yield of the plasmid. Using Equation 1.0 and Equation 2.0, it has been shown that the QIAGEN plasmid purification midi kit procedure yielded approximately 5 $\mu$ g purified pGEX-6P-1-InlA plasmid total, 8.0 ng of which was transformed into One Shot BL21(DE3)pLysS *E. coli* cells for expression of the InlA-GST fusion protein.

The purified plasmid pGEX-6p-1-InlA was successfully transformed into the BL21 cells (Figure 3.0 A,B). The BL21(DE3)pLysS expression competent *E. coli* cells grew on LB agar containing ampicillin which directly suggests that the pGEX-6p-1-InlA, which confers ampicillin resistance (AmpR), was present in the BL21 cells when plated onto the LB agar. If the ampicillin resistant plasmid had not been successfully transformed into the cells, no colonies would have grown in the presence of the ampicillin. The BL21 cells also grew in the presence of chloramphenicol, which directly suggests that the pLysS plasmid was also present in the cells, since this plasmid carries a gene for chloramphenicol resistance (CamR). The presence of pLysS is important because it expresses the T7 lysozyme needed to lower background expression level of InlA before it is needed. The DE3 lysogen carried by BL21(DE3)pLysS strain expresses

a T7 RNA polymerase under the control of lacUV5 promoter, so that only after IPTG is added will expression of InlA-GST commence (Invitrogen, 2010). In this way I was able to control when InlA-GST expression would occur.

BL21 transformation with 10 pg control ampicillin resistant pUC19 plasmid was carried out in order to ascertain a transformation efficiency of  $3 \times 10^6$  transformants per  $\mu\text{g}$  plasmid DNA, which is slightly less than the expected transformation efficiency for BL21(DE3)pLysS of  $10^8$  transformants per  $\mu\text{g}$  of supercoiled plasmid (Invitrogen, 2010). Un-transformed BL21 cells plated onto LB agar plates containing no antibiotics formed a dense lawn of bacterial growth which suggests that there was no phage contamination in the BL21 cultures. Un-transformed BL21 cells plated onto LB agar containing ampicillin (50 $\mu\text{g}/\text{ml}$ ) did not form any colonies, because while the inherent pLysS plasmid confers chloramphenicol resistance, the BL21 cells did not contain any plasmid which would bestow ampicillin resistance, such as the pGEX-6p-1-InlA or the pUC19 plasmid. However, the un-transformed BL21 cells did in fact grow colonies on LB agar containing chloramphenicol (34 $\mu\text{g}/\text{ml}$ ), showing that the pLysS plasmid was present in the BL21 cells and that the T7 lysozyme would be expressed, lowering the background expression level of InlA-GST until the appropriate time.

Finally, Expression of InlA-GST fusion protein was stimulated by adding IPTG to liquid cultures of BL21(DE3)pLysS-pGEX-6P-1-InlA-1 *E. coli*, activating the lacUV5 promoter and T7 RNA polymerase. The cultures were centrifuged and the cell pellets were frozen at -20°C until bacterial lysis and protein extraction could be carried out. Freezing the cell pellets also aids in the subsequent lysis process. Purification of the InlA-

GST fusion protein from the cell pellet will need to be carried out before the expression of InlA-GST can be absolutely confirmed. Thereafter Western blotting can be applied.

The future steps in our study will be to purify the InlA-GST protein from the BL21 cells using glutathione affinity based chromatography. The InlA protein contains a GST tag, so a Pierce GST Spin Purification Kit will be used to isolate the InlA-GST. The GST will then be cleaved from the InlA. The GST will remain bound to the glutathione resin column while the InlA will filter through, yielding purified InlA protein. The InlA will then be fluorescently labeled with Alexa Fluor dye and bound to different sizes of microbeads in varying densities by passive adsorption.

The InlA functionalized beads will then be used to study the phagocytosis process in several epithelial cell line cultures in order to compare the effects of ligand density, bead size, and eukaryotic target cell versatility. The endocytosis process and kinetics will be analyzed by flow cytometry and fluorescent microscopy for each cell line to compare internalization rates. It has already been shown that internalization of InlA functionalized beads can be achieved in at least some epithelial eukaryotic cell lines (Blanchette et al., 2009). We are therefore applying the protocols in this previous study and expanding on this idea by demonstrating that this method can be applied to a vast range of epithelial cells. In doing so, we can ensure that this method of intracellular delivery can be used consistently among different individual cell types and could be a promising efficient drug delivery option in the future. The implications of our study are great, because it is important to study the means by which pathogens infect cells in order to attempt treatment.



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